REGULATION OF THE SYNTHESIS OF NUCLEOTIDE PHOSPHOHYDROLASE AND NEUTRAL DEOXYRIBONUCLEASE: TWO ACTIVITIES PRESENT WITHIN PURIFIED VACCINIA VIRUS

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Abstract.—Regulation of the synthesis of viral nucleotide phosphohydrolase and neutral DNase was investigated by means of DNA and protein inhibitors. These virus-associated activities, previously localized within viral cores, are introduced into host cells with the infecting inoculum. Nucleotide phosphohydrolase was found throughout the cycle of multiplication in a particulate fraction containing viroplasmic matrices. However, the DNase from the inoculum appeared in the supernatant fraction, and the DNase synthesized after infection was associated with the particulate material. The two enzymatic activities belong in the category of "late" functions. It now appears that during morphogenesis of the poxviruses several "late" proteins, some with enzymatic properties, are enclosed within the viral envelopes.

Introduction.—New enzymatic activities appear in animal cells after infection by the poxviruses. At least four of these activities are also present in highly purified virus particles.[1-4] Some have been designated as "early functions" that can be transcribed from parental DNA genomes; others, the "late functions," require DNA replication prior to their appearance.[5]

Among the four activities found to date in the viral cores, the RNA polymerase[6] and acid DNase-exonuclease[7, 8] belong in the late category, the neutral DNase-endonuclease was regarded as an early function,[9] while information bearing on this point regarding the nucleotide phosphohydrolase[2] was lacking. In the present study, dealing with the regulation of the synthesis of the latter two activities, it was established that both the neutral DNase and phosphohydrolase are late functions. It now appears likely that, as morphogenesis of the poxviruses proceeds, several late proteins, some with enzymatic properties. are integrated within the virus progeny during maturation.

Materials and Methods.—The IDH strain of vaccinia virus and L_2 cells were used in accordance with experimental procedures described previously.[10]

For synchronous infection 10 plaque-forming units/cell were allowed to adsorb in suspension at 4°C for 1 hr. Unadsorbed virus was removed by washing and centrifugation. The virus-cell complexes were dispensed into 100 mm Petri dishes, each plate receiving 10^7 cells. Uninfected control cell cultures were prepared in an identical manner.

DNA was labeled with ^{3}H-methyl-thymidine (spec. act. 11 C/mM) at a concentration of 0.5 μc/ml and protein with a L-amino acid-^{14}C (uniformly labeled) mixture at a concentration of 1 μc/ml, added to nutrient medium from which unlabeled amino acids were omitted but a supplement of 2% bovine fetal serum was added.

The incorporation of the isotope was stopped after exchanging tracer-containing medium with unlabeled medium supplemented with 10% serum. Adherent cells were released from plates with a rubber policeman, then centrifuged into pellets, resuspended in a solution of 0.25 *M* sucrose and 10 mM KCl, and disrupted in a Dounce homogenizer by

applying 10–20 strokes with a tight-fitting pestle. The nuclei, released intact and generally free of cytoplasm, were sedimented at 800 × *g* for 5 min and discarded. The supernatant was centrifuged at 10,000 × *g* for 15 min; the resulting pellets, termed "particulate fraction," and the supernatant were used for enzymatic assays. In some experiments the particulate fraction was mixed with sodium deoxycholate (DOC). To remove membranous organelles and debris, the mixture containing 1% DOC in Tris-HCl buffer pH 7.4, for each milligram of protein, was centrifuged again into pellets at 10,000 × *g* for 15 min. In other experiments, DOC extraction was omitted and the particulate fraction, suspended in Tris-HCl buffer, was layered on sucrose gradients. Continuous sucrose gradients were preformed by mixing 20% and 50% sucrose solutions in Tris-HCl buffer pH 8.3 and 1 mM $MgCl_2$. The material was spun to equilibrium at 50,000 rpm for 90 min in a SW65 rotor and separated into 20 fractions collected dropwise through the bottom of the tube.

DNase activities were determined as described previously.[4] ATP-γ-^{32}P (sp. act. 1.1 C/mM and 980 mC/mM) was used as substrate for the nucleotide phosphohydrolase assays.[2] Protein concentrations were estimated by the method of Crampton *et al.*[11]

The source of inhibitors and other compounds was as follows: the antibiotic Rutamycin (Eli Lilly Co.) was provided by Dr. A. Tzagoloff. Ouabain (Strophanthin-G), hydroxyurea, and cytosine arabinoside HCl were purchased from Sigma Chemical Co., and sodium deoxycholate from Mann Research Laboratories, Inc. Streptovitacin A was a gift from the Upjohn Co.

Results.—Characterization of nucleotide phosphohydrolase activity in a particulate fraction from infected cells: Since animal cells contain at least two ATPases, it was necessary to differentiate between them and the viral phosphohydrolase activity identified previously in vaccinia particles. For this purpose, two kinds of experiments were performed. In the first series, a particulate fraction was obtained from infected cells that had been labeled with ^{14}C-amino acids and ^{3}H-methyl-thymidine. After centrifugation to equilibrium in continuous sucrose gradients, this material could be separated into predominantly viral and cellular components. A fraction of the cytoplasmic material labeled in the DNA for 2.5 to 3.5 hours after infection banded coincidentally with the bulk of the labeled protein synthesized 5 to 6 hours after infection (Fig. 1). Labeled DNA and protein which remained near the top of the gradient were observed in extracts of

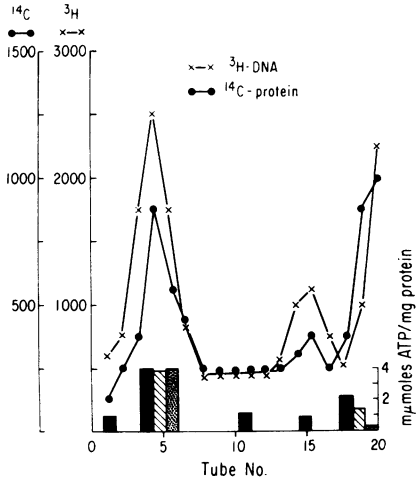

FIG. 1.—Separation of the nucleotide phosphohydrolase activity after centrifugation through sucrose gradients. Cultures inoculated with 10 PFU/cell were pulse-labeled separately either with ^{3}H-methyl-thymidine at 1.5–2.5 hr post infection (p.i.) or ^{14}C-amino acids at 5.5–6.0 hr p.i. Both cultures were harvested at 6 hr p.i., and prepared for density gradient centrifugation. Fractions of equal density were collected sequentially from 3 gradients and then pooled and adjusted to the required pH and $MgCl_2$ concentration. Activity was measured as the hydrolysis of ATP-γ-^{32}P for 1 hr at 37°.[2]

Aliquots were taken for determination of the protein content in each sample. Bar graph: Filled bars, activity as mμmoles of ATP-γ-^{32}P hydrolyzed/hour/mg of protein; bars with diagonal lines, activity as above in the presence of 10^{-3} *M* ouabain; bars with dots, as above in the presence of 10 μg/ml of Rutamycin.

both infected and uninfected cells, whereas the denser DNA-protein band was absent from the control material. This result indicates that vaccinia-specific DNA and protein were being separated by the gradient. Hydrolysis of ATP-γ-^{32}P was monitored in selected samples from the gradients, as indicated in Figure 1. Although ATPase activity was detected throughout the gradient, the highest activity was present in fractions where the dense band of DNA and protein occurred. Furthermore, in this fraction the phosphohydrolase was refractory to inhibition by Rutamycin and ouabain, indicating that neither the mitochondrial nor membrane ATPases were involved. On the other hand, ATPase hydrolysis by material trapped near the top of the gradient was almost entirely inhibited by Rutamycin and ouabain. Results with these inhibitors are in agreement with the data (mentioned previously) regarding the presence near the top of the gradient of cellular DNA and protein after centrifugation of cytoplasmic material from uninfected cells.

In the second series of experiments the cytoplasmic particulate fraction was treated with DOC to remove membranous contaminants. This procedure released 45 to 65 per cent of the protein originally present in the particulate fraction. After treatment with DOC, the residual material was spun once again into pellets and used for nucleotide phosphohydrolase assays or electron microscopic examination. Enzyme activity was also tested simultaneously in particulate and supernatant fractions that had not been subjected to DOC extraction. Data from these experiments, summarized in Table 1, revealed, as expected, background ATPase activity in both particulate and supernatant fractions from the uninfected controls. There was also a twofold increase of the phosphohydrolase activity in pellets obtained at six hours from infected cultures. Exposure of the particulate material to DOC profoundly reduced the background ATPase in the controls but had no appreciable effect on material from infected cells (Table 1). There was no obvious difference in ATP hydrolysis by supernatant fractions from control and infected cells. These experiments demonstrated an increase with time of an ATPase activity that was resistant to DOC treatment and specific to virus-infected cells. Further differentiation between cellular and viral ATPase activity in the DOC-treated pellets was achieved with ouabain and Rutamycin. When 10 μg/ml of Rutamycin were added to the

TABLE 1. *Nucleotide phosphohydrolase activity in particulate and supernatant cytoplasmic fractions.*

Hours after infection		mμM ATP-γ-^{32}P Hydrolyzed per mg Protein in 1 Hr		
		Particulate fraction	Particulate fraction after DOC	Supernatant
0	Virus	6.4	2.5	4.0
	Control	5.4	1.0	5.0
3	Virus	6.3	5.3	5.4
	Control	5.5	3.0	5.0
6	Virus	11.0	10.0	4.3
	Control	5.5	2.5	5.1

The pellets prepared as in Fig. 6 were resuspended in Tris-HCl buffer 0.01 *M* pH 7.3 and divided. One fraction was used for enzymatic assays and protein determination; the other treated with DOC (0.5% final concentration) was spun again into pellets at 10,000 $\times$ *g* for 15 min, resuspended in buffer. and then assayed for ATPase activity and protein content. Activity was expressed as in Fig. 1.

incubation mixture containing material from cells infected for six hours, ATP hydrolysis was reduced only 15 per cent, but was inhibited 84 per cent in comparable fractions from uninfected cells. Addition of 10^{-3} *M* ouabain plus 5 mM Na^+ and K^+ failed to inhibit activity in both control and infected material, indicating an absence of the membrane-associated Na-K ATPase that must have been lost as a consequence of treatment with the detergent. A slight contamination of the particulate fraction from infected cells by mitochondrial debris is indicated by these observations.

The morphological character of the DOC-extracted particulate fraction was ascertained by thin sections in the electron microscope. The material within pellets prepared six hours after infection consisted predominantly of viroplasmic matrices. More than half such matrices contained profiles of immature progeny particles (Fig. 2). However, these particles lacked their characteristic membranes,[10] which were probably damaged or destroyed because of exposure to

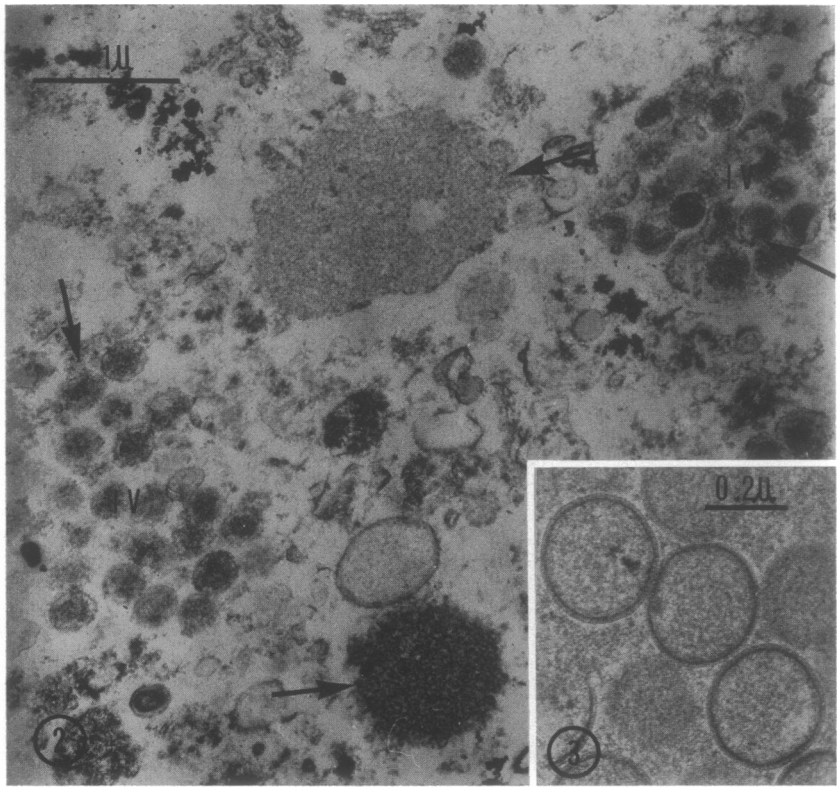

FIG. 2.—Thin section through a pellet prepared from DOC-treated particulate fraction. Arrows point toward aggregates of viroplasmic matrix, some enclosing profiles of immature vaccinia particles (*IV*). The viral membranes became indistinct following exposure to the detergent. ×23,000.

FIG. 3.—Thin section through several vaccinia particles arrested in their morphogenesis at an immature stage as a result of treatment for 12 hr of infected cultures with 5×10^{-3} *M* hydroxyurea. The DNA nucleoids, normally present inside the membrane, are missing from these particles. × 64,000.

DOC. The pellets also contained mature vaccinia particles and unidentifiable amorphous or membranous debris, presumably of cellular origin.

Regulation of viral nucleotide phosphohydrolase synthesis: Since the predominant phosphohydrolase activity could be localized in the particulate fraction from infected cells, it became possible to determine whether vaccinia DNA had to be replicated to obtain the increment in enzyme activity that was observed during a normal cycle of infection. For this purpose we used cytosine arabinoside and hydroxyurea at concentrations that effectively (95–98%) inhibited the incorporation of labeled thymidine into DNA. It was previously established,[12] and confirmed by us, that hydroxyurea arrests the development of vaccinia at an immature stage, characterized by particles possessing viral membranes (Fig. 3), indicating that at least some viral proteins are produced in the absence of DNA replication. However, when either inhibitor was added to the cultures immediately after adsorption of the virus, the nucleotide phosphohydrolase activity remained at the level present soon after inoculation (Figs. 4 and 5).

In experiments employing streptovitacin A to inhibit protein synthesis, it was found that an increase of enzyme activity with time was also blocked effectively (Table 2). Taken together, these data indicate that *de novo* synthesis of the viral nucleotide phosphohydrolase requires a prior synthesis of DNA and, therefore, this activity by definition fits into the category of a "late" function.

Regulation of viral DNase (endonuclease) synthesis: Among the four virus-contained enzymatic activities identified to date, three had been characterized as late functions. The pH 7.8 DNase, classified previously as an early function,[9] seemed to be exceptional. This discrepancy was checked by studying the appearance of this DNase activity in infected cultures when DNA and protein synthesis were suppressed. The cytoplasmic component derived from homogenized cells was further separated into particulate and supernatant fractions and

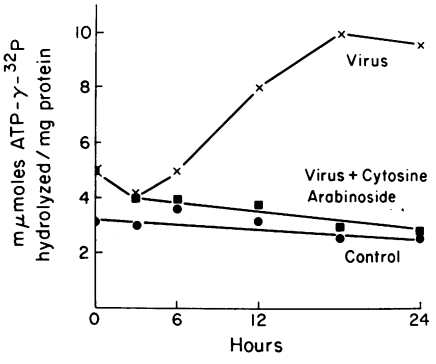

FIG. 4.—Effect of cytosine arabinoside on the induction of nucleotide phosphohydrolase activity following infection. Enzymatic activity was assayed in the DOC-treated particulate fraction as indicated in Table 1. To inhibit DNA synthesis the cultures received 20 μg/ml of the analogue.

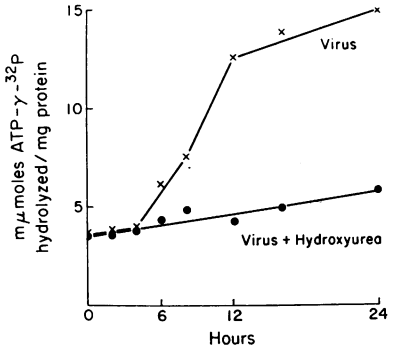

FIG. 5.—Effect of hydroxyurea on the induction of nucleotide phosphohydrolase activity following infection. DNA synthesis was inhibited by final concentrations of 5×10^{-3} *M* hydroxyurea in the culture medium. Assays were conducted as in Table 1 and Fig. 4. Data are average values from two independent experiments.

TABLE 2. *Effect of streptovitacin A on the nucleotide phosphohydrolase activity following infection.*

Hours after infection	mμM ATP-γ-^{32}P Hydrolyzed per mg Protein in 1 Hr		
	Virus	Virus + streptovitacin	Control cells
0	3.7	...	2.8
3	1.5	1.5	1.0
6	4.0	2.0	2.0
16	10.0	2.0	...
24	16.0	4.0	4.0

Activity was tested at various times in the DOC-treated particulate fraction as described in the legend of Table 1. 10 μg/ml of streptovitacin A were added to the cultures, beginning 1 hr after inoculation.

both of these were monitored for activity. The data summarized in Figure 6 revealed that within two hours after inoculation there was a very significant elevation of activity in the 10,000 *g* supernatant without a corresponding increase in the particulate fraction. This supernatant DNase activity was found also when DNA and protein inhibitors were added. The activity decayed with time to the same low level as in the uninfected controls. On the other hand DNase activity in the pellet fraction increased progressively, beginning four to six hours following inoculation. This increase was prevented by treatment of cultures with either hydroxyurea or streptovitacin A. Therefore the pH 7.8 DNase is also synthesized as a late protein.

Discussion.—The dependence on DNA replication for synthesis of the two enzymatic activities examined in this study indicates that they belong in the category of late functions. Therefore, all four activities that have been identified to date within poxviruses are most probably coded from progeny DNA templates. These late proteins are then packaged inside viral envelopes.

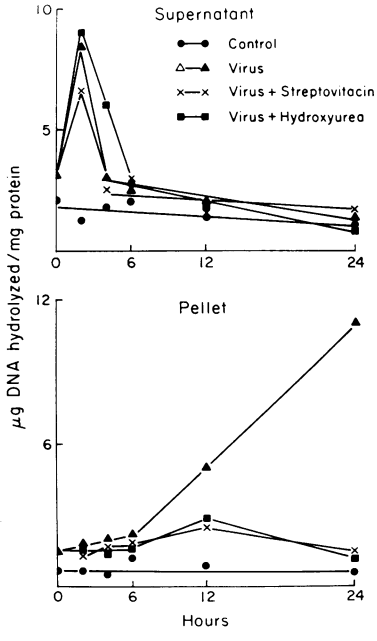

FIG. 6.—Effect of hydroxyurea and streptovitacin A on the induction of pH 7.8 DNase activity. DNase activity was tested in both the particulate and the supernatant of cytoplasmic fractions from infected and uninfected L_2 cells. Cytoplasmic extracts were centrifuged at 10,000 × *g* for 15 min. The resulting pellets were resuspended in Tris-HCl buffer pH 7.8 with 20 mM 2-mercaptoethanol (2-ME) and 1% Nonidet P_{40} (NP_{40}). The remaining supernatant fraction was adjusted to pH 7.8 (Tris-HCl 0.05 *M*) and contained 2-ME and NP_{40}. The DNase activity is expressed as μg of DNA rendered acid-soluble in 1 hr per mg protein. Concentrations of 5 × 10^{-3} *M* hydroxyurea or 10 μg/ml streptovitacin A were used. The data are average values from two independent experiments.

However, the morphogenesis of the envelopes is an expression of early viral functions, since they can be produced when DNA synthesis is blocked by a variety of drugs, including hydroxyurea,[12] as illustrated in Figure 3, or when transcription and translation are stopped three to four hours after infection.[10] Apparently the proteins that are incorporated during the maturation process include the RNA polymerase, nucleotide phosphohydrolase, and two DNases. The mechanism by which these late proteins are integrated into vaccinia progeny particles remains to be elucidated.

Although synthesis of the enzymatic activities associated with the particulate fraction, and ultimately with the virus progeny, begins relatively late during the infectious cycle, these activities are also introduced into the host with the inoculum. Therefore, the infected cell acquires them from the invading particles and also synthesizes them during the infectious cycle. The nucleotide phosphohydrolase, one of the activities examined in this study, was identified only in the particulate fraction (Table 1), indicating that it remains bound to the viral core after penetration. On the other hand, the pH 7.8 DNase of the inoculum is active predominantly in the supernatant fraction (Fig. 4), presumably because it is released from the core as a smaller entity. However, after the initiation of *de novo* synthesis, beginning about six hours after infection, DNase activity in the particulate fraction increases progressively, while activity in the supernatant decays rapidly. Release of the DNase from inoculum particles into the soluble fraction could be the natural mechanism for activating DNases of the invading virus after it enters into the cytoplasmic matrix. The significance of this event and the possible rôle of vaccinia-associated DNases were discussed previously.[4] At least three explanations can be offered to account for the relatively low increment in the DNase detected in the particulate fraction (Fig. 4). Most probably the particulate fraction contains an inhibitor that was shown to exist in vaccinia particles,[4] while in the supernatant the DNase of the inoculum is separated from this inhibitor and thereby becomes more active. This has been substantiated by preliminary unpublished experiments. Secondly, since a high multiplicity is used to infect synchronously, the ultimate yield of progeny particles may be only an order of magnitude higher than the number added as the inoculum. Thirdly, some progeny particles are released after maturation from the infected cell into the medium. These three factors either singly or in combination could explain the low activity of the induced pH 7.8 DNase.

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